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EFFECT OF NUCLEOTIDES ON POTENTIAL AND pH CHANGES ACROSS THE THYLAKOID MEMBRANE OF SPINACH CHLOROPLASTS

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Summary

With appropriate preparations of spinach chloroplasts we observe three distinct effects of the nucleotides:

1. An accelaration of the dark decay of the light induced 520 nm absorbance change after ATP addition.
2. An acidification of the internal space of the thylakoids after ATP addition in darkness.
3. A dark ATPase activity wich is regulated by the ΔpH across the membrane.

We conclude that the effect of the nucleoside triphosphates on the 520 nm signal is linked to a change of the proton conductivity of the membrane, induced by the formation of a ΔpH across the membrane in consequence of the dark ATPase activity.

The mode of action of the nucleoside diphosphates in the presence of inorganic phosphate on the 520 nm signal is discussed. It is proposed that the effects observed are linked to the hydrolysis of the newly formed nucleoside triphosphates.

Introduction

By illumination of a chloroplast suspension a potential and a pH difference are generated through the thylakoid membrane. These two properties are usually characterized respectively by an absorbance change at 520 nm [1] and a quenching of the 9-aminoacridine fluorescence [2].

In tightly coupled chloroplasts phosphorylation accelerates the decay of the 520 nm absorbance change [3—4] and decreases the stationary level of this absorbance change during continuous illumination [5].

Abbreviations: CF_1, coupling factor 1; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine.

It is generally thought that these effects are the consequence of the faster dissipation of the proton electrochemical potential linked to the phosphorylation rate of ADP into ATP by the intermediary of the coupling factor 1 [6—7] in accord with the chemiosmotic hypothesis [8].

We previously reported that the acceleration of the dark decay of the 520 nm absorbance change was initiated as well by ATP as by ADP plus inorganic phosphate (P_i). We proposed [6] that either added ATP or newly formed ATP from ADP plus P_i modify the membrane properties and by way of consequence the kinetics of the 520 nm signal decay.

In this study we intend to focus on the changes which are brought about in the chloroplasts by ATP addition in the darkness. For this purpose the dark decay of the light induced 520 nm absorbance changes and the pH changes "inside" the thylakoid membranes will be investigated. The effects of the nucleotides on these two properties are shown to be governed by an ATPase activity, dependent on the coupling factor 1 (CF_1).

Materials and Methods

1. Chloroplasts preparation

Freshly harvested spinach leaves were illuminated for half an hour in ice cold water. Chloroplasts were rapidly isolated using a standard technique [9] in 0.4 M sucrose, 0.05 M Tris at pH 7.8, 0.01 M NaCl, then washed once in 0.4 M sucrose and finally resuspended in 0.4 M sucrose, 1% bovine serum albumin and kept in this medium in ice at concentration equivalent to 3 mg of chlorophyll per ml.

2. Spectrophotometric measurements

The absorbance changes at 520 nm were measured in a medium 0.4 M sucrose, 5 mM $MgCO_3$, 10 mM tricine at pH 8.3, 10 μM phenazine methosulphate and chloroplasts equivalent to 17 or 35 μg of chlorophyll per ml.

The temperature was 5° C. The 520 nm absorbance change during continuous illumination was tested to be sensitive to valinomycin in the presence of K^+ ions and insensitive to uncoupling concentrations of methylamine (3 mM).

The flash induced 520 nm absorbance changes were measured as before [6]. For each experiment, the decay kinetics of the absorbance changes were decomposed into a rapid and a slow exponential phase. Acceleration of the dark decay of the 520 nm absorbance change leads to a decrease of the amplitude of this slow phase.

The 520 nm absorbance change during continuous illumination was recorded with a double beam spectrophotometer Perkin Elmer 356 or Aminco DW-2 in the dual mode (520 nm minus 540 nm). The sample was contained in a 10 mm × 10 mm (3 ml) cuvette, thermostated at 5° C, and submitted to 6 sequences of 10 s light and 10 s dark periods. The signal was averaged on the 5 last periods. The photomultiplier was protected by an optical filter (MTO Specivex DH 485 b) and the actinic light was filtered through a Kodak Wratten filter 92 and a Calflex B_1 (light intensity 2 mW/cm^2).

The uptake of the 9-aminoacridine was measured by its fluorescence quenching. The reaction mixture was 0.4 M sucrose, 5 mM $MgCO_3$, 20 mM KCl when

present, 10 mM tricine at pH 8.3, 3.3 μM 9-aminoacridine and chloroplasts equivalent to 17 or 35 μg of chlorophyll per ml. The temperature was 5° C. The fluorescence corresponding to a ΔpH of zero was measured in the presence of 10 mM methylamine.

The fluorescence of 9-aminoacridine was determined with a Jobin et Yvon spectrofluorimeter (type Beam). The fluorescence at 470 nm was observed at right angle of the excitation light at 380 nm. The photomultiplier was protected by an optical filter (MTO Specivex DH 485 b). The sample was thermostated at 5° C in a 10 mm × 10 mm cuvette (3 ml).

3. Biochemical assays

ATPase activity was measured by the release of ^{32}P from [γ-^{32}P]ATP as well as by the acidification of the medium after ATP addition. In the first method, in a final volume of 0.1 ml (0.4 M sucrose, 5 mM $MgCO_3$, 10 mM tricine at pH 8.3), [γ-^{32}P]ATP was incubated at 5° C. The reaction was started by addition of chloroplasts (2 μg of chlorophyll). After 5 min, the reaction was stopped by addition of 0.04 ml 2 N HCl. The inorganic phosphate was separated from ATP by high voltage electrophoresis [10] and the radioactivity was measured with a Nuclear Chicago gas flow counter. In the second method in a final volume of 30 ml (0.4 M sucrose, 5 mM $MgCO_3$, 20 mM KCl) chloroplasts, equivalent to 0.5 mg of chlorophyll, were continuously stirred in a thermostated cuvette at 5° C and the pH adjusted to pH 8.2 with 2.5 mM NaOH or 2.5 mM HCl by means of a two-way Radiometer pH-stat. The reaction was started by additon of a known amount of ATP and monitored during 5 min.

The ΔpH through the thylakoid membrane was determined either from the distribution of [^{14}C]methylamine between the internal and the external volume [11] or from the fluorescence of the 9-aminoacridine [2]. The reaction mixture for the assay of [^{14}C]methylamine uptake was 5 mM $MgCO_3$, 20 mM KCl when present, 20 mM tricine at pH 8.2, 0.25 mM methylamine (^{14}C-labelled, 38 Ci/mol or unlabelled), 0.5 mM sorbitol (^{14}C-labelled 97 Ci/mol or unlabelled), ^3H_2O ($2.5 \cdot 10^{-6}$ Ci per ml) and chloroplasts equivalent to 125 μg of chlorophyll per ml. Temperature was 5° C. After 5 min standing the pellets in 0.2 ml were spun down with a microfuge Beckmann through 0.1 ml of a silicone fluid layer consisting of 55/45 w/w of versilube F.50/fluide silicone SF 96/50, into 0.2 ml of an aqueous solution (8.5% of glycerol, 1.5% of trichloractic acid) following the method of Portis and McCarty [12]. ^3H and ^{14}C activities were determined with a liquid scintillation system (Mark III Searle Analytic Inc.).

ATP synthesis was measured by following the rate of incorporation of ^{32}P from $^{32}P_i$ into ATP, after electrophoretic separation of ATP from P_i. The assay was run in the same medium as for 520 nm signal measurement but with 0.4 mM ADP, 0.8 mM P_i and chloroplasts equivalent to 10 μg of chlorophyll per ml. Temperature was 25° C.

4. Chemicals

Nucleotides were purchased from Sigma Chemical Company, [γ-^{32}P]ATP, 475 Ci/mol, D-[U-^{14}C]sorbitol, 97 Ci/mol from the Radiochemical Center Amersham, [^{14}C]methylamine, 38.4 Ci/mol and ^3H_2O 10^{-5} Ci/mol from CEA

IRE SORIN. Dio-9 was kindly given by Gist Brocades Research and Development and FCCP was a gift of Dr. P.G. Heytler.

Versilube F.50 from General Electric Silicones and Fluid Silicone SF.96/50 were purchased from Ravaud et Muscadet.

The immunoglobulin against CF_1 was prepared from rabbits [13] by the laboratory of Enzymology of CNRS (91190 Gif-sur-Yvette, France).

Results and Discussion

1. Effect of ATP addition on the dark decay of the 520 nm signal

We have studied the effect of nucleotides on:

(a) The amplitude of the slowly decaying portion of the absorbance change at 520 nm, induced by a group of flashes.

(b) The stationary level of the 520 nm absorbance change during continuous illumination.

The dark decay of the flash-induced 520 nm signal decomposes into two exponential decaying portions, a fast ($t_{1/2}$ 5—10 ms) and a slowly decaying ($t_{1/2}$ 100—200 ms) portions. Fig. 1 shows that after ATP addition the magnitude of the slowly decaying portion was lowered and that ADP had an antagonistic effect. The resulting effect of ATP was an acceleration of the dark decay of the 520 nm signal. Addition of ADP plus inorganic phosphate was as effective as addition of ATP.

During continuous illumination the stationary level of the 520 nm signal is governed, for a given light intensity, by the rate of the dark decay. Fig. 2 shows that the magnitude of this 520 nm signal was decreased by ATP addition as well as by ADP plus P_i addition and restored by increasing concentrations of ADP at a fixed ATP concentration.

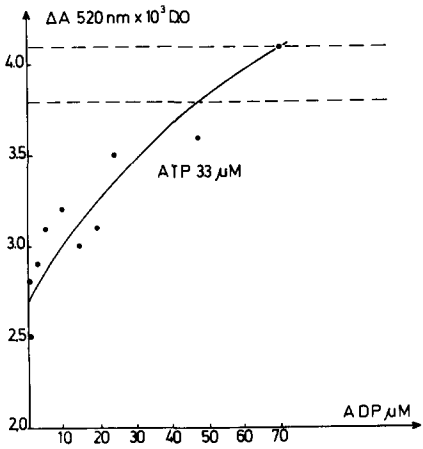

Fig. 1. Antagonistic effect of ADP on the acceleration by ATP of the dark decay of the flash induced 520 nm signal. The amplitude of the slowly decaying portion of the absorbance change at 520 nm, induced by a group of flashes, is plotted versus the concentration of ADP. In the medium (given in Methods), chloroplasts equivalent to 17 μg of chlorophyll per ml are excited by groups of six flashes either in the absence of ATP (the dashed lines delimit the variations of the control measurements) or with 33 μM ATP added without ADP or concomitantly with various concentrations of ADP.

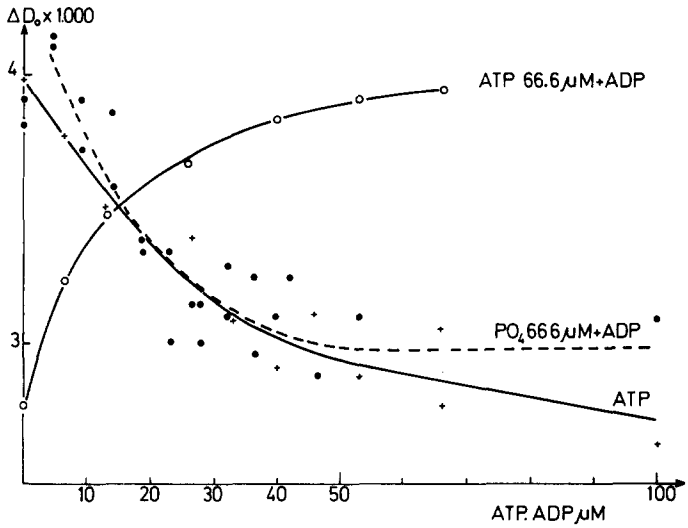

Fig. 2. Effect of nucleotides on the magnitude of the stationary level of the 520 nm signal (520—540 nm). In the medium (given in Methods), with chloroplasts equivalent to 35 μg of chlorophyll per ml, the stationary level of the absorbance at 520 nm minus absorbance at 540 nm is measured at different concentrations of ATP (+) or ADP plus 666 μM P_i (•) or 66.6 μM ATP in the presence of various concentrations of ADP (○).

Although it is difficult to separate the light and dark processes in these experiments we interpret the above results as the effect of ATP (added or newly formed) in dark reactions.

2. Effect of ATP addition on the pH difference through the thylakoid membrane

When ATP was added in the chloroplast suspension in the dark the fluorescence of the 9-aminoacridine was partially quenched (Fig. 3). The maximum quenching was around 30 to 50% of the initial fluorescence. It was specific for ATP; AMP and ADP were ineffective. Moreover, ADP reversed the effect of ATP (Fig. 3). The quenching of the 9-aminoacridine fluorescence is interpreted as a probe of the formation of a pH difference through the thylakoid membrane [2].

We verified this ΔpH formation by measurement of the [^{14}C]methylamine distribution between the osmotic water and the medium following the method developped by Rottenberg et al. [11]. After 5 min standing in darkness with ATP there was a reproducible and significant accumulation of [^{14}C]methylamine inside the membranes which is usually interpreted as an acidification of the internal space (Table I).

The results obtained by these two methods are qualitatively identical but quantitatively different. Both point out to an increase of the ΔpH after ATP addition (the pH inside being lower than the pH outside) and to the presence of a ΔpH even in control chloroplasts. In the case of 9-aminoacridine the level of the fluorescence corresponding to a null ΔpH was observed in the presence of methylamine or FCCP.

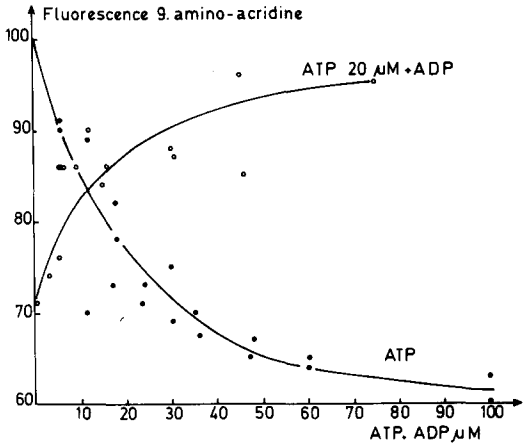

Fig. 3. Effect of the addition of ATP or ADP (in the presence of ATP) on the level of the fluorescence of 9-aminoacridine in chloroplast suspensions kept in the dark. In the medium (given in Methods) chloroplasts equivalent to 17 μg of chlorophyll per ml are suspended in the dark in the presence of 9-aminoacridine. The medium is supplemented with ATP (•) or with ADP in the presence of 20 μM ATP (○). The fluorescence level is expressed in arbitrary units: 100 corresponds to the level of fluorescence of 9-aminoacridine when chloroplasts are incubated in the dark without any nucleotide addition.

The same acidification of the internal space was induced by the newly formed ATP. This was shown by experiments in which chloroplasts were illuminated 1 min in the presence of ADP plus P_i and then kept in the dark for 5 min. After this time, which allows a complete decay of the light dependent membrane potential and reversible ΔpH, we indeed found an acidification of the osmotic space (0.3 to 0.6 unit of pH).

3. Dark ATP hydrolysis induces the pH difference through the membrane

The ATPase activity of our chloroplasts in absolute darkness was tested by following either the acidification of an unbuffered medium with a pH-stat (Fig. 4) or the release of $^{32}P_i$ from [γ-^{32}P]ATP after the two compounds

TABLE I

ΔpH THROUGH THE THYLAKOID MEMBRANE INDUCED BY 300 μM ATP ADDITION IN COMPLETE DARKNESS

Conditions were those in Methods, but 9-aminoacridine fluoresecence was measured with chloroplasts equivalent to 125 μg of chlorophyll per ml as for the [^{14}C]methylamine technique.

Expt.	Addition	Measurement with			
		[^{14}C]methylamine		9-aminoacridine	
		Without ATP	With ATP	Without ATP	With ATP
1		1.04	1.16	2.27	2.79
2		1.34	1.51	2.08	2.75
3		1.43	1.55	2.04	2.75
4		1.06	1.5	2	2.75
5	K^+ + 5 μM valinomycin	0.71	1		

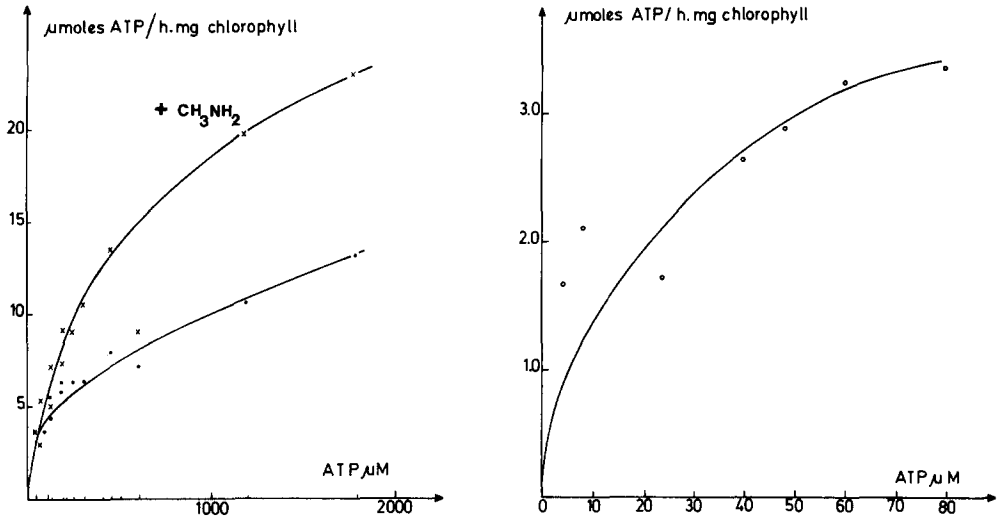

Fig. 4. Saturation curve of the ATPase activity of a chloroplast suspension by medium acidification. In the medium (given in Methods) chloroplasts equivalent to 17 μg of chlorophyll per ml, are incubated at 5°C and the pH is kept constant at 8.2 by means of a pH-stat in the dark. The reaction is started by addition of ATP. For each concentration of ATP, the reaction is monitored during 5 min in the absence of methylamine (•) and then in the presence of 10 mM methylamine (X) added 5 min after ATP.

Fig. 5. Saturation curve of the ATPase activity of a chloroplast suspension measured by release of $^{32}P_i$ from [γ-^{32}P]ATP. Experimental conditions are given in Methods but chloroplasts are equivalent to 17 μg of chlorophyll per ml.

were separated by high voltage electrophoresis (Fig. 5). It appears that an ATPase activity is already present in the dark.

When an uncoupler, like methylamine, was added after ATP addition in the dark the ATPase activity was enhanced (2 to 5 times) but when methylamine was added before ATP addition, the ATPase activity was inhibited (Table II). Addition at the same time of methylamine and ATP led to an ATPase activity which was only three times that of the control.

Valinomycin plus potassium showed a similar effect but at a smaller extent (Table II).

TABLE II

EFFECT OF METHYLAMINE OR VALINOMYCIN ADDITION ON THE ATPase ACTIVITY OF THE CHLOROPLASTS IN THE DARK

ATPase activity was measured with the pHstat. Conditions were as described in Methods but chloroplasts were equivalent to 200 μg of chlorophyll per ml and ATP was 2 mM.

Addition	μmol · h^{-1} · mg^{-1} chlorophyll
2 mM ATP	15
10 mM methylamine 5 mn after 2 mM ATP	70
10 mM methylamine with 2 mM ATP	37
10 mM methylamine 3 mn before 2 mM ATP	7
2.5 μM valinomycin 5 mn after 2 mM ATP	43
2.5 μM valinomycin with 2 mM ATP	25
2.5 μM valinomycin 3 mn before 2 mM ATP	15

In the presence of uncoupler, the ΔpH through the membrane is limited or null. We infer that a less value of ΔpH has two effects on the ATPase activity:

(a) ATPase activity is enhanced because the accumulation of protons, inside the membrane, which results from the ATP hydrolysis, is rapidly dissipated.

(b) ATPase activity is inhibited because the ΔpH through the membrane is a prerequisite for the activation of the ATPase.

Of these two antagonistic effects one or the other will be dominant, depending on the order of addition of ATP and methylamine.

If methylamine is added in the same time or after ATP, the first effect overcomes the second. ATPase activity is enhanced and sustains by itself a sufficient ΔpH to maintain the ATPase in its active form.

If methylamine is added before ATP, the ΔpH is suppressed and ATPase activity cannot develop. The active form of the ATPase reverses to an inactive one.

The effect of valinomycin plus potassium can be related to the partial ΔpH inhibition (Table I) which was observed in their presence.

In the chloroplasts, where an active ATPase is present, we found:

(a) a ΔpH through the membrane even in darkness and in the absence of nucleotides (Table I);

(b) in the presence of ATP, dark hydrolysis leads to an acidification of the internal osmotic space. That proton translocation represents an "energization" of the membrane induced by hydrolysis of ATP and has been already observed in chromatophores [14] as in vesicles of *Escherichia coli* [15]. In these organisms the energy transduction in the complex membrane system is reversible as also in mitochondria. This reversibility has also been found in chloroplasts after induction of ATPase activity either by light treatment in the presence of thiol compounds or by trypsin action [16—22].

We propose that the ATPase activity we have found in chloroplasts is subjected, as Bakker-Grunwald has shown for the light triggered ATPase activity in the presence of dithiothreitol or trypsin, to an energy-linked equilibrium between active and inactive conformation [23]. The ΔpH through the chloroplast membrane induces activation of the ATPase. ATP hydrolysis leads to a proton translocation which maintains a ΔpH through the membrane and by way of consequence keeps the ATPase in its active form.

4. Relationships between dark ATPase activity and nucleotide effects on the 520 nm signal and the 9-aminoacridine fluorescence

The dark ATPase activity depends on numerous factors and we investigate the influence of changes of its intensity on the nucleotide effects in the chloroplasts.

(a) ATPase activity changes depending on the state of the chloroplasts. From freshly harvested spinach leaves which are afterwards illuminated in iced water for half an hour, we isolate chloroplasts where ATPase is active. The reproducibility of the results depends largely on this period of preillumination. Kraayenhof et al. [24] have reported that, in intact chloroplasts ("class 1"), ATPase activity is already present without any activation process. Our chloroplasts are type B in Hall's nomenclature [25]. Ferricyanide penetrates freely in these chloroplasts, as also nucleotides.

TABLE III

RESTORATION BY PREILLUMINATION OF THE ATPase ACTIVITY AND OF THE QUENCHING OF THE 9-AMINOACRIDINE FLUORESCENCE BY ATP ADDITION

ATPase measurement was done as in Table III but after 20 s of illumination in red light (4 mW/cm^2) followed by 100 s of darkness before ATP was added. Fluorescence measurements were done as described in Methods but after 20 s of red light (4 mW/cm^2) 9-aminoacridine was added and after 100 s of darkness ATP was mixed with the chloroplasts, final concentration 30 μM.

Chloroplast treatment	Fluorescence quenching (%)	ATP hydrolyzed μmol · h^{-1} · mg^{-1} chlorophyll
Fresh chloroplasts	46	6
Aged chloroplasts	9	1.8
Aged chloroplasts after preillumination	32	5.4

The isolated chloroplasts, stored in 0.4 M sucrose in the presence of 1% bovine serum albumine at 0° C, kept their activity for several hours. Then the ATPase activity dropped rapidly. When chloroplasts were isolated from fresh leaves collected during cloudy periods and the preilluminations was omitted, their ATPase activity was very low or null. The ATPase activity could be restored in both cases by a short illumination of the chloroplasts (20 s) followed by a dark period of 100 s before ATP addition (Table III).

ATP addition led to a small or null quenching of the 9-aminoacridine fluorescence in these chloroplasts where the ATPase activity was low or zero (Table III). But the short illumination (20 s) as above restored ATP effect on the 9-aminoacridine fluorescence as well as the ATPase activity.

With these chloroplasts which exhibited a low ATPase activity before to be illuminated, we obtained only a very small acceleration by ATP of the dark decay of the 520 nm signal after the first flash group. After the second flash group ATP was more effective and the maximum effect was observed after the fifth group (Fig. 6). The same pattern was obtained by replacing ATP by ADP plus P_i. With the same type of chloroplast and the same flash technic, we verified that the ATPase activity was progressively restored, depending on the number of flash groups received (Fig. 7). Therefore the acceleration by ATP or ADP plus P_i of the dark decay of the flash induced 520 nm signal parallels this ATPase activity restoration and the parallel ΔpH formation.

(b) Changes of the hydrolysis rate in relation to the concentration of ATP added. Fig. 8 shows that in the range of 0 to 100 μM, increasing concentrations of ATP causes a parallel augmentation of the rate of ATP hydrolysis, a decrease of the stationary level of the 520 nm signal and a quenching of the fluorescence of the 9-aminoacridine.

Beyond this concentration range (Fig. 4) there is a slower increase of the ATPase activity which is linearly related with the ATP concentration. Increasing concentration of ATP above that limit does not cause any further changes of the 520 nm signal and of the 9-aminoacridine fluorescence emission even if the amount of hydrolysed ATP does increase. We infer that ATP effect on the 520 nm signal is parallel to the ATPase activity only when the rate of ATP hydrolysis is proportional to the extent of the ΔpH formed or the quenching

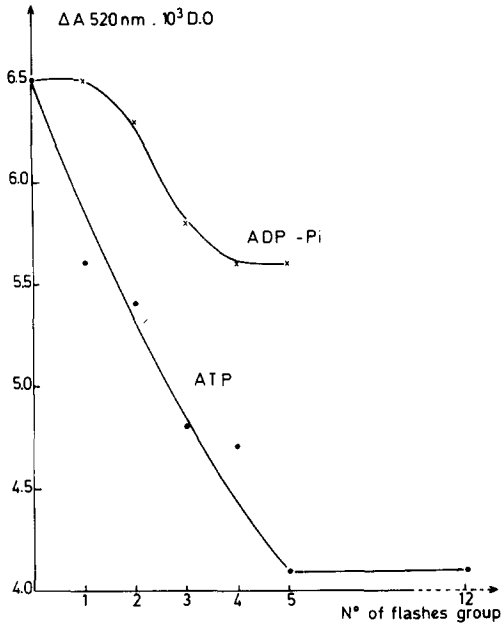

Fig. 6. Effect of the number of exciting flash groups on the acceleration by ATP or ADP plus P_i of the dark decay of the flash induced 520 nm signal. Chloroplasts, equivalent to 17 μg of chlorophyll per ml, are suspended in the medium, given in Methods, with 333 μM ATP (•) or 333 μM ADP plus 666 μM P_i (X). The suspension is submitted to series of flash groups at a frequence of 4 per min. After each flash group the dark decay of the 520 nm signal is monitored. The corresponding slowly decaying portion of the signal is plotted as a function of the number of flash groups the chloroplasts have received. The results are the average of four repetitions.

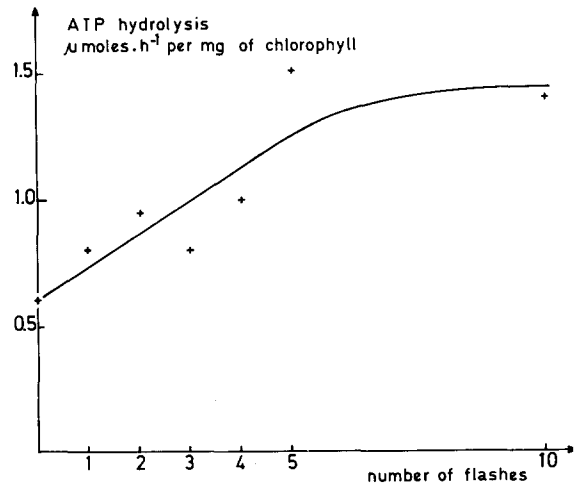

Fig. 7. Effect of the number of exciting flash groups on the ATPase properties of aged chloroplasts. Chloroplasts equivalent to 250 μg of chlorophyll per ml are suspended in the medium given in Methods with 70 μM [γ-^{32}P]ATP. The suspension is submitted to series of flash groups with the same technic and frequence as in Fig. 6 but with white light. The reaction is stopped after 3 min.

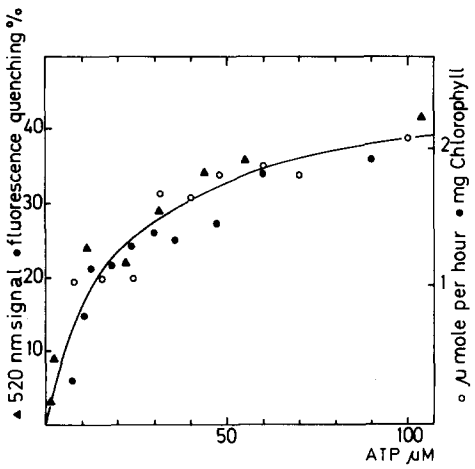

Fig. 8. Effect of ATP concentration on the rate of ATP hydrolysis, decrease of the stationary level of the 520 nm signal and quenching of the fluorescence of the 9-aminoacridine. Conditions of the experiments are respectively these indicated in the legends to Figs. 5, 2 and 3. The rate of ATP hydrolysis is expressed in $\mu mol \cdot h^{-1} \cdot mg^{-1}$ of chlorophyll. The decrease of the 520 nm signal and of the fluorescence of the 9-aminoacridine are expressed in percent of the total signal or total fluorescence of the control.

of the 9-aminoacridine fluorescence which measures this ΔpH. As pointed out by Schonfeld and Neuman [26] the proton conductance through the membrane apparently does not increase until a certain value of this ΔpH, but above this threhold it is increasing drastically. The higher the proton conductance, the faster the membrane potential, which is formed by illumination of the chloroplasts, will decrease. In the presence of an ATPase activity the ΔpH through the membrane in the dark is already larger than this threshold and the dissipation of the membrane potential (whose rate is measured by the rate of the dark decay of the light-induced 520 nm absorbance change) is faster than in the absence of any ATPase-like process.

(c) Changes of the hydrolysis rates in relation to the nature of the nucleoside triphosphate. The different nucleoside triphosphates were hydrolyzed in the dark at different rates, and represented a different effectiveness as for decreasing the level of the 9-aminoacridine fluorescence and the extent of the 520 nm absorbance change in continuous illumination (Table IV). The specificity of the nucleotides for these effects was the same as that which has been reported for light-induced and light-triggered ATPase activity in chloroplasts [27—28] or for the Ca^{2+}-dependent ATPase activity of the heat- or trypsin-treated CF_1 [28—29]. In the case of etheno-ATP we measured a rate of hydrolysis which was always one fifth of the rate of ATP hydrolysis. Etheno-ATP had no effect on the 520 nm signal and the 9-aminoacridine fluorescence intensity (Table IV).

As we have previously shown [6] for ATP and ADP in the presence of inorganic phosphate, the dark decay of the 520 nm absorbance change was in any case accelerated almost identically by nucleoside triphosphates or by the corresponding nucleoside diphosphate in the presence of inorganic phosphate.

TABLE IV

QUENCHING OF THE 9-AMINOACRIDINE FLUORESCENCE IN DARKNESS AND INHIBITION OF THE 520 nm ABSORBANCE CHANGE DURING STEADY STATE ILLUMINATION, BY NUCLEOSIDE TRIPHOSPHATE ADDITION

Conditions were as these reported in Methods but nucleotides were 67 μM and chloroplast concentration was equivalent to 17 μg of chlorophyll per ml.

Addition	9-aminoacridine fluorescence quenching (%)	Inhibition of the 520 nm absorbance change (%)
ATP	45	32
GTP	17	12
ITP	7	7
UTP	1	4
CTP	0	2
etheno-ATP	5	1

(d) Inhibition of the ATPase activity. The effects of ATP on the 520 nm signal and on the fluorescence of the 9-aminoacridine were inhibited at the same extent by a given concentration of Dio-9. Dark ATPase activity appeared to be less sensitive to Dio-9 and ATP synthesis, during cyclic photoreactions, was still more resistent (Figs. 9 and 10). Interpretation of these differences of sensitivity is not simple because Dio-9 alone decreases the extent of the 520 nm signal and enhances the fluorescence emission of the 9-aminoacridine. These effects lead us to suppose that Dio-9 induces some changes of the membranes properties which could not directly be related to the energy transfer process.

ADP is an inhibitor of the ATPase activity and the Fig. 11 shows that the same degree of inhibition was observed at the same concentration of ADP for the ATP hydrolysis and for the ATP effects either on the 520 nm signal or on the 9-aminoacridine fluorescence emission. In Fig. 11, the inhibitory effect of ADP was not as complete as in Figs. 1—3. The experiments reported there were done with summer chloroplasts which were less sensitive to ADP than the winter chloroplasts which were used for the experiments in Figs. 1—3.

All these results stress the strong correlation between the effect of the nucleoside triphosphate on the 520 nm signal and its rate of hydrolysis or more preciseley the extent of the ΔpH which is concomitantly formed.

The necessary connection between the ability of the chloroplasts to hydrolyse the nucleoside triphosphates and the effect of the nucleotides on the 520 nm signal holds strong enough when the nucleoside diphosphate plus inorganic phosphate are added instead of the corresponding nucleoside triphosphate. This connection is evident during the restoration, by successive flashes, of the ATPase activity as well as the comparison of the effects on the 520 nm signal of the addition of the different nucleotides. The most prominent confirmation is the lack of effectiveness of etheno-ADP plus P_i [6] on the 520 nm signal. Shahak et al. [28] have indeed shown that etheno-ADP is phosphorylated at almost the same rate as ADP, but that etheno-ATP is a very poor substrate for the ATPase, although it binds to CF_1.

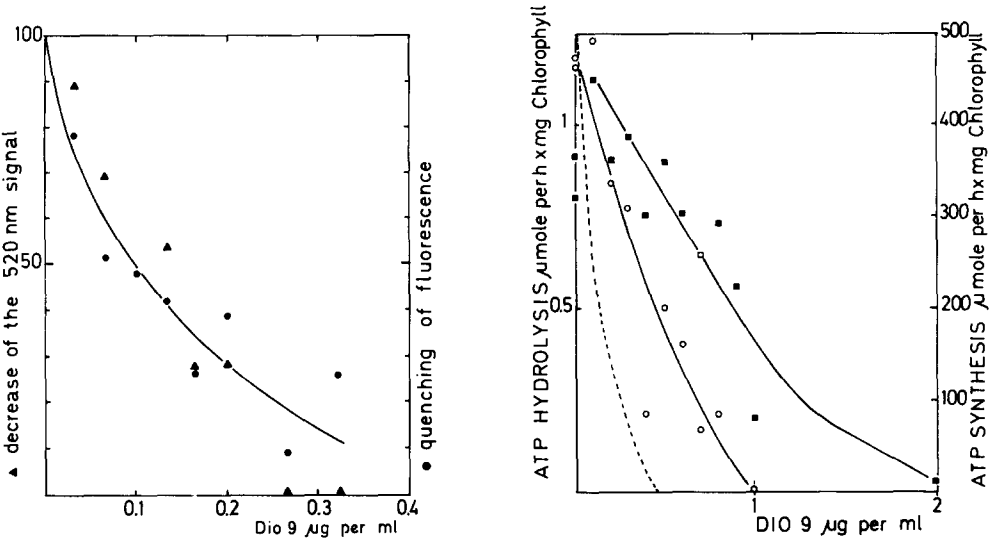

Fig. 9. Inhibition of the effect of ATP on the 520 nm signal and on the fluorescence of the 9-aminoacridine by Dio-9. Conditions are respectively these of the legends to Figs. 2 and 3 but chloroplasts are equivalent to 30 µg of chlorophyll per ml and 19 µM ATP is added. The decrease of the 520 nm signal and the quenching of the 9-aminoacridine fluorescence, induced by ATP addition are expressed in % of the corresponding measurements done in the absence of Dio-9.

Fig. 10. Effect of Dio-9 on ATP hydrolysis and ATP synthesis. ATP hydrolysis (○) and ATP synthesis (■) are measured as indicated in Methods but chloroplasts are equivalent to 30 µg of chlorophyll per ml, 20 µM ATP for ATP hydrolysis and 1 µM ADP plus 2 µM P_i for ATP synthesis. The two results are expressed in $\mu mol \cdot h^{-1} \cdot mg^{-1}$ of chlorophyll. We have reported in dotted line the curve of Fig. 9. In this last case 100% corresponds to the graduation 500 on the right side of the Fig. 10.

5. *Effect of immunoglobulin against* CF_1

After treatment of the chloroplasts with immunoglobulin against CF_1 the acceleration of the dark decay of the flash induced 520 nm absorbance change

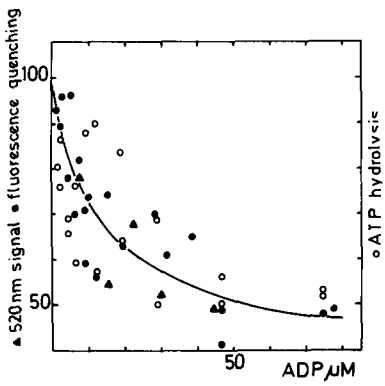

Fig. 11. Inhibition of the effect of ATP on the 520 nm signal, the fluorescence of the 9-aminoacridine and the ATP hydrolysis, by ADP addition. Conditions are these reported in Fig. 8 but ATP is 32 µM. The decrease of the 520 nm signal and quenching of the fluorescence of the 9-aminoacridine caused by ATP addition as the rate of the hydrolysis of ATP are expressed in percent of the corresponding measurements done in the absence of ADP. For ATP hydrolysis 100 corresponds to 1.5 μmol hydrolyzed $\cdot h^{-1} \cdot mg^{-1}$ of chlorophyll.

TABLE V

EFFECT OF THE IMMUNOGLOBULIN ON THE RATE OF PHOTOPHOSPHORYLATION AND ON THE DECREASE OF THE AMPLITUDE OF THE SLOW PHASE OF THE 520 nm SIGNAL BY ADP PLUS P_i AND ATP ADDITION

Conditions were those described in Methods but nucleotides were 0.4 mM and P_i 0.8 mM. 100% photophosphorylation correspond to 411 μmol per hour and mg of chlorophyll of P_i esterified.

mg of anti-CF_1 per mg of chlorophyll	Photophosphorylation rate	Decrease of the amplitude of the slow component of the 520 nm absorbance change by addition of	
		ADP + P_i	ATP
0	100	100	100
1	95	83	87
2	59	21	68
3	37	0	50
4	24	0	11

by nucleotide addition was inhibited. The same treatment inhibited the phenazine methosulfate mediated photophosphorylation. But the immunoglobulin inhibited more drastically the acceleration of the dark decay of the flash induced 520 nm absorbance change in the presence of ADP plus P_i than in the presence of ATP (Table V).

These results show that CF_1, as expected, is implicated in the nucleotides effects on the 520 nm signal. But we think they help to decide between the two interpretations given by Saphon et al. [30] and Baltscheffsky [31]. These authors have reported an ATP effect on the 520 nm signal respectively in *Rhodopseudomonas sphaeroides* and *Rhodospirillum rubrum* chromatophores, but their interpretations are at variance. For the former, ATP is hydrolyzed and the ADP and P_i formed are reacting back to give ATP in a reaction which dissipates the proton electrochemical potential and causes an acceleration of the dark decay of the 520 nm signal. For the later this acceleration is the consequence of the "energization" of the chromatophore membranes by ATP, either added or formed from ADP and P_i.

The results of our experiments done in the presence of immunoglobuline against CF_1 confirm the interpretation given by Baltscheffsky. In Baltscheffsky's interpretation the effect of ATP is directly linked to the ATPase activity and ADP plus P_i effect to the rate of hydrolysis of the formed ATP. This last rate of hydrolysis is dependent on the rate of phosphorylation of ADP and on the ATPase activity so is governed by two successive reactions inhibited by immunoglobuline. The effect of ADP plus P_i addition should be more sensitive to immunoglobuline than the effect of ATP addition. It is what we observed.

It therefore seems that the acceleration of the dark decay of the 520 nm signal by addition of nucleoside diphosphates in the presence of inorganic phosphate is not controlled by the rate of the phosphorylation of the nucleotide but dependent on the ability of the chloroplasts to hydrolyse the formed nucleoside triphosphate.

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